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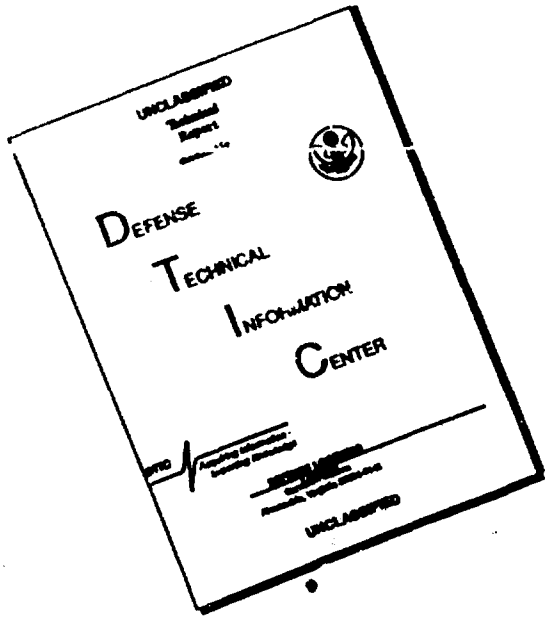

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KINETICS OF THERMAL INACTIVATION OF TRYPSIN SOLUTIONS IRRADIATED WITH ULTRAVIOLET LIGHT

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Our first report described results obtained from a study of the kinetics of denaturation of natural trypsin. In this portion of the work carried out under analogous conditions we were investigating the kinetics of thermal inactivation of trypsin solutions exposed to ultraviolet light.

Materials and methods

Trypsin solutions [1] with a concentration of 2.0 mg/ml in 10^{-3} n. H_2SO_4 (or HCl) were exposed to full light from a PRK-2 bulb equipped with a thermal filter. During irradiation the cell with the solution (layer thickness of 0.5cm) was set in ice and washed with a mixture of ethyl alcohol and water chilled to -2°. The distance from the cell to the bulb was 17cm. Following irradiation the solutions were mixed in a 1:1 proportion with 10^{-3} n. H_2SO_4 (or HCl) and heated to the desired temperature in a thermostat. Before determining enzyme activity the samples were kept at 1.5 - 2.0° for no less than 40 minutes.

Experiment results

As distinct from unaltered trypsin, trypsin exposed to ultraviolet light and heated under conditions indicated is not inactivated according to the law $A = A_0 e^{k\tau}$ where A_0 is the specific activity of the solution immediately after irradiation; A is the current specific activity; in both cases the enzymatic activity is related to the total protein concentration in the solution. Since we used only the proportion A/A_0 the differences are not found in what concentration to attach total activity to.

Kinetic inactivation lines in semilogarith coordinates had a break

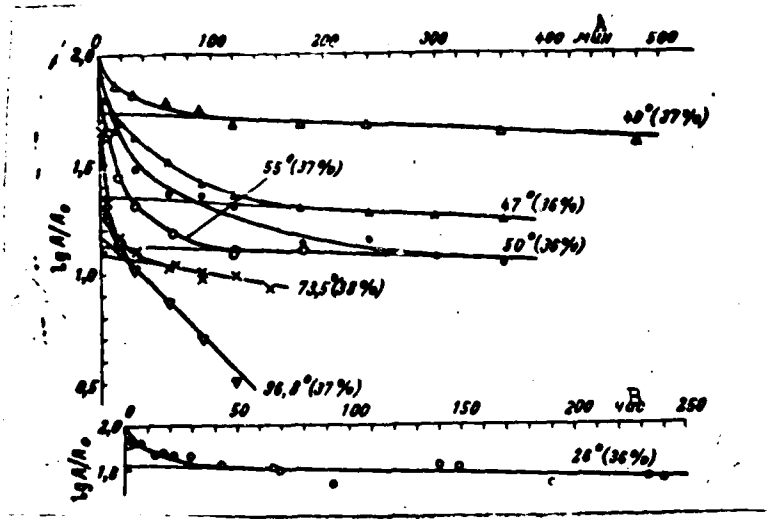

Fig. 1 Inactivation of trypsin irradiated with ultraviolet light at different temperatures (in parentheses is given the relative specific activity immediately after irradiation)
Key: A - minutes B - hours

and a linear section toward the end. In Fig. 1 we have data on post-irradiation inactivation of trypsin exposed to ultraviolet light at different temperatures. The irradiation dose was the same in all cases as indicated by the close values for the relative specific activity of enzyme solutions following irradiation (Fig.1). For temperatures of 50, 55, 73.5 and 96.8° the final linear segments extrapolatedto $\tau = 0$ intersect the y axis at approximately the same point. Their slopes correspond to constants for the rates of thermal denaturation of natural trypsin at the same temperatures. For this reason we feel that the intersection points indicated give the fraction of natural protein in the irradiated solution. At lower temperatures (26, 40, 47°) the intersection points of extrapolated end segments with the y axis lie much higher and their slopes exceed the values which must correspond to the denaturation of natural trypsin [1].

The heating to 96° of an ultreviolet-irradiated trypsin solution which has been kept for a long time at 26° produces rapid inactivation of the enzyme. The general depth of the process corresponds quite well to data obtained from direct thermal inactivation (96°) of the same dose of irradiated trypsin (Fig.2).

Thermal inactivation of irradiated trypsin solutions was irreversible. Prolonged incubation at a low temperature (+3°) did not produce reactivation. Thus the size of the specific enzyme activity did not increase in a heat-inactivated solution, if activity was measured 24 hours after being kept at

+3°, as compared with specific activity measured after a 40-minute incubation at the same temperature after heating.

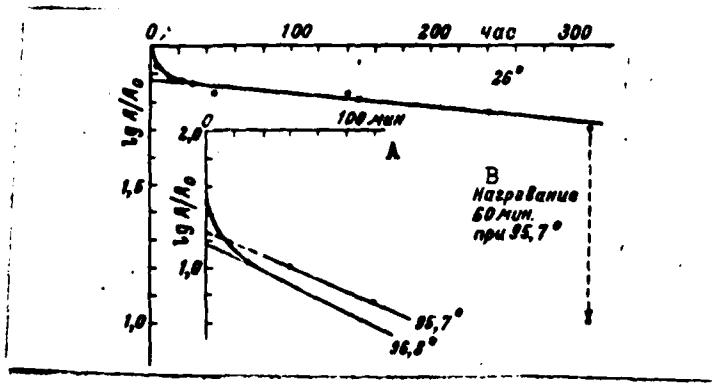

Fig. 2 Kinetics of inactivation of trypsin irradiated with ultraviolet light.
c equals 1.0 mg/ml in 10^{-3} n.H_2SO_4. Residual activity after irradiation 35%. Line obtained at 96.8° corresponds to the inactivation of trypsin immediately following irradiation. Points at 95.7° correspond to enzyme inactivation after 314 hours of incubation of the irradiated solution at 26°.
Key: A - minutes
B - heating 60 minutes at 95.7°

Discussion of results

As we can see from the previous exposition [1], the kinetics of denaturation of natural trypsin can not be described by the simple diagram of N⟶D and for this reason the kinetic laws of the inactivation of an irradiated enzyme by heating are more complex than those described for other situations with proteins [2, 3]. In order to elucidate kinetic features observed let us consider several hypotheses.

1. Following prolonged storage (low temperatures) a portion of the potentially damaged trypsin is reconverted into natural trypsin.

2. Following irradiation all constants in the kinetic diagram of the denaturation process are modified. There is first of all a reduction (or loss) of the capacity of trypsin to pass from the reversibly denatured form into the active form.

The first hypothesis can be disposed of immediately. In Fig. 2 we can see that the heating of irradiated trypsin which has been kept for a long time at 26° leads to a rapid loss of enzymatic activity. The depth of this process corresponds essentially with a case in which a solution irradiated with

the same dosage was heated immediately afterward to a high temperature (96.8°). If there had been a conversion of potentially damaged trypsin into natural protein the rate of inactivation would had to have been less since natural protein must lose its activity under the same conditions more slowly than irradiated protein [1].

Let us write down a diagram for the inactivation of potentially damaged trypsin analogous to that used for the inactivation of natural trypsin [1]:

$$D'_H \xleftarrow{k'_3} R \underset{k'_2}{\overset{k'_1}{\rightleftharpoons}} D_0$$

(R is the trypsin with latent damage) and let us find out how applicable this is for R). It has been shown that for natural protein (N) the relation between the constants for the rates of the corresponding stages is this: $k_1 \gg k_3$ and $k_2 \gg k_3$, i.e. the reversible stage of denaturation takes place much faster than the irreversible [1]. For this reason at high temperatures (above 50°) and with a longer heating time (up to 10 minutes) trypsin is found predominantly in a reversibly denatured form D_0 and at low (approximately 0°) in natural form N. The question arises as to whether the ratio between constants is maintained for R too or whether there is a reverse ratio: $k'_3 \gg k'_1$ and $k'_3 \gg k'_2$, i.e. when irradiated protein is heated the $R \longrightarrow D'_H$ process predominates. Finally, there is reason to assume [4] that trypsin with potential damage does not pass from the inactive form D'_0 into the active form N, i.e. $k'_2 \approx 0$. These problems are considered below.

Kinetics of the inactivation of ultraviolet-irradiated trypsin solution (a case of several fractions inactivated according to an exponential law)

A Удельная доза облучения, тыс. эрг/мм²	B Температура, °С	C Константа скорости инактивации k, сек⁻¹ при содержании активных фракций в смеси после облучения, % D										
		0	7	10	14	25	27	37	39	58	60	70
37.0	96.8	$1.9 \cdot 10^{-3}$	$6.0 \cdot 10^{-3}$	—	E Нативный	—	—	—	—	—	—	$3.3 \cdot 10^{-4}$
40.0	73.5	—	—	—	»	—	$3.0 \cdot 10^{-3}$	—	—	$1.9 \cdot 10^{-3}$	—	—
40.0	73.5	—	—	—	»	$1.6 \cdot 10^{-3}$	—	—	—	—	$1.7 \cdot 10^{-3}$	—
37.0	55.0	—	—	—	»	$7.0 \cdot 10^{-4}$	—	—	—	—	$4.5 \cdot 10^{-3}$	—
35.0	50.0	—	—	—	»	$2.3 \cdot 10^{-4}$	—	—	—	—	$1.9 \cdot 10^{-3}$	—
35.0	47.0*	—	—	$5.8 \cdot 10^{-4}$	»		—	$4.45 \cdot 10^{-4}$	$6.1 \cdot 10^{-4**}$	—	—	—

* At 47° the kinetic line moves slowly toward a slope corresponding to the denaturation of natural protein at this temperature. For this reason in processing it is is presumed that the number of latent-damage molecules is the same here as at higher temperatures.

** The value cited in this chart is for orientation since there were few experimental points in the initial segment of the kinetic line.

(Key follows on next page)

Key: A - specific activity following irradiation o/o.
B - temperature in C
C - Inactivation rate constant k, sec.$^{-1}$
D - when active fractions are kept in a mixture after irradiation,%
E - natural

It has been shown that egg albumin which in the natural form denatures according to the simple pattern of $N \longrightarrow D$ consists of several fractions after irradiation [2]. An analysis of the kinetic lines of posteffects has made it possible to determine denaturation rate constants (k_1) for separate fractions and their activation energy. We also attempted to present kinetic inactivation lines in the form of a sum of several exponents. The results of our calculations are given in the table above. Let us direct our attention to the following facts. In the first place, between 50 and 73.5° the number of fractions and their proportion in the mixture are constant. In the second place, for the majority of molecules in latent-damage trypsin (60%) at this temperature range the activation energy of the post-radiation inactivation process is 19±2 kilocalories/mol and for the minority (26%) 21±2 kilocalories/mol (Fig.3). Both these values are close to the activation energy obtained for irreversible inactivation of the natural protein ($N \xrightarrow{k_3} D_H$). The latter equals 22 kilocalories/mol [1]. At the same time, a considerable amount of data [2; 3] gives reason to believe that following irradiation the energy of activation of thermal denaturation must drop substantially. Hence we should conclude that an activation energy of 19±2 kilocalories/mol or 21±kilocalories/mol does not belong to the process $R \xrightarrow{k_3'} D_H'$. The inactivation of latent-damage trypsin corresponds rather to the process $R \xrightarrow{k_1} D_0'$ since for the process $N \xrightarrow{k_1} D_0$ (N is natural enzyme) the activation energy is 75±8 kilocalories/mol which is considerably greater than the values cited for R [1]. From the table cited it follows that at 47 and 96.8° the fraction makeup of irradiated trypsin varies. Fraction composition as a function of temperature may be related to the difference in activation energies for the denaturation of different fractions. Such a situation is illustrated in Fig. 4. Here the values T and k are arbitrary. In the temperature range $T_1 - T_2$ many fractions have similar inactivation rate constants. For this reason the number of fractions identifiable in a given interval of temperature may be small. It is clear that the activation energy in this interval has some sort of mean value between energies E_i characteristic of individual fractions, i.e. $E_i \gtrsim 19 \pm 2$ kilocalories/mol.

It was shown above that in the case of latent-damage trypsin as well as in the case of the natural enzyme $k_1' \gg k_2'$. It remains for us to explain how constant k_2 changes after irradiation. Let us assume that k_2' has a conspicuous value. In the simplest case when k_3 is small the kinetic inactivation lines must have limits corresponding to the position of equilibrium $R \rightleftharpoons D_0'$, the position of which depends on temperature [1]. If the solutions heated at different temperatures are cooled to 1.5 - 2.0° we must reduce the activity to a constant level corresponding to the values for constants k_1' and k_2' at low temperature. If $k_1' \gg k_3'$ and $k_2' \gg k_3'$ but k_3' is of appreciable

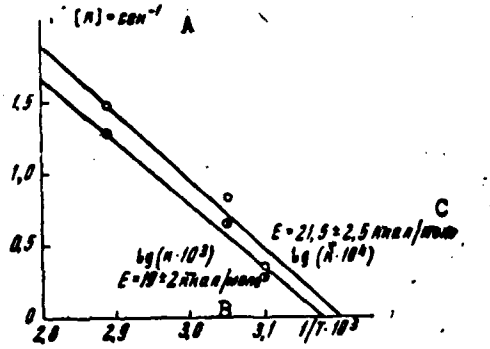

Fig. 3 Logarith of the constant of inactivation rate as a function of 1/T for different fractions in ultraviolet-irradiated trypsin
Activation energy 10±2 kilocalories/mol corresponds to the inactivation of the major fraction
Key: A - [k] equals sec^{-1}
B - E equals 19±2 kilocalories/mol
C - E equals 21.5±2.5 kilocalories/mol

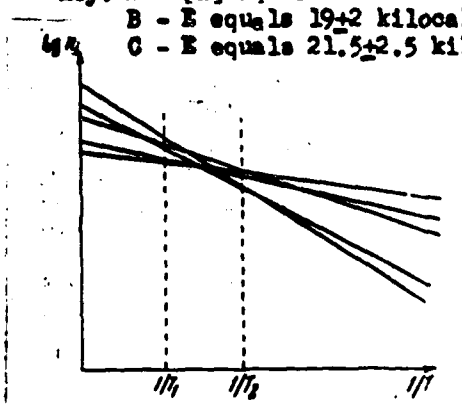

Fig. 4 Schematic diagram of the logarithm of the constant of the monomolecular reaction in enzyme inactivation as a function of 1/T.
Case of many fractions distinguishable by thermolability. Each straight line corresponds to one fraction.

value, this may be due to rather small observation times when an equilibrium can be successfully established and irreversible denaturation does not make any real contribution to the process. We assume that in our experiments this moment may correspond to the break in the line [1]. Experimental data indicate that this feature does not develop (Fig.1). The situation of the breaks in the lines does not depend on temperature. At high temperatures the depth of the inactivation process is greater than at low temperatures. In addition, measurment of enzyme activity in a heated solution kept in an ice

bath for 40 minutes, several hours or days gives identical results. These facts give reason for assuming that $k_1' = 0$ or $k_1' \approx 0$, i.e. trypsin with latent damage does not possess the ability for denaturation in a reversible fashion when subjected to heat. It is probable that this in related to a rupture in one of the six disulfide bonds in the protein molecule [4].

The hypothesis exists [3] that the transition to the latent-damage state occurs when a certain number of hydrogen or other bonds in the protein molecule are ruptured. This number may be determined from the reduction in the activation energy of thermal denaturation of the enzyme following irradiation. It is obvious that such an approach is possible only in a case where the reaction follows a single reaction course both before and after irradiation. A drop in activity in an irradiated trypsin solution is connected to the transition $R \xrightarrow{k_1'} D_0'$. Under these condition natural trypsin is inactivated according to the pattern $N \xrightarrow{k_1} D_H$, i.e. the two processes take place according to different patterns. The transition of R and N to the activated state is accompanied by a rupture of different bonds. Hence a comparison of activation energies for these processes does not give us any basis for conclusions as to the nature and number of bonds which rupture from irradiation in natural trypsin molecules and lead to their transition to the latent-damage state. This obviously applies to other proteins as well where denaturation passes through reversible and irreversible stages.

Conclusions

1. The kinetic features of thermal inactivation of ultraviolet-irradiated trypsin solutions may be explained if we take into account that the latent-damage enzyme loses its capacity for reversible denaturation.

2. Thermal inactivation processes of natural and ultraviolet-irradiated trypsin follow different reaction patterns under identical conditions.

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